

Rational Design and Directed Evolution of Human Paraoxonase I (huPON1) for Increased
Solubility and Stability in *E. coli*

A Senior Honors Thesis

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By

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Abstract

Paraoxonase I (PON1) is a serum hydrolase that has been found to hydrolyze organophosphates (OP), such as VX and sarin. Because human PON1 (huPON1) is a human protein with reasonable activity, it seems like a promising therapeutic agent against OP poisoning. However, huPON1 is an insoluble protein and not very stable when expressed in *E. coli*, whereas a chimeric mammalian PON1 variant, G2E6, has proven to be soluble and expressible in *E. coli*. We are specifically working on a way to make huPON1 more soluble by using a rational approach of changing a cluster of hydrophobic residues on the surface of the protein into polar ones. Since these mutations are on the surface of the protein, it is rationalized that it may not significantly affect the activity of huPON1.

We are employing two different rational designs to make huPON1 more soluble. The first strategy utilizes a three fragment PCR overlap containing 12 hydrophobic residues from the HDL binding site that are mutated to polar residues. The second design utilizes total gene synthesis in order to make 16 hydrophobic to polar residues in huPON1 corresponding to the G2E6 mutations that are polar. These variants were put into a vector containing folding reporter GFP to screen for solubility and expression by means of cellular fluorescence. We have been able to express both variants and have observed them to be significantly more soluble than the wild-type one.

In order to achieve even more solubility, we have fused these huPON1 variants with Maltose Binding Protein (MBP) which has been shown to improve the solubility of many proteins. By doing this, we have been able to express and purify large amounts of protein in order to obtain biophysical characteristics. Both huPON1 variants prove to be active against paraoxon and phenyl acetate.

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Introduction

Organophosphate Background

Organic phosphorous (OP) compounds have been developed for their effectiveness as insecticides. However, because the mammalian and insect cholinergic nervous systems are similar, these compounds have been responsible for millions of poisonings and thousands of deaths annually.¹ These OP compounds target acetylcholinesterase, a β -esterase that hydrolyzes acetylcholine, a neurotransmitter, into acetate and choline.¹ If acetylcholinesterase is inhibited, acetylcholine will accumulate in the synaptic clefts, causing a cholinergic over-stimulation.¹ This over-stimulation leads to cholinergic syndrome which includes increased sweating and salivation, bronchial secretion, bronchoconstriction, increased gastrointestinal motility, tremors, muscular twitching, and other central nervous system activities.¹ Because of these known toxic affects, OP derivatives have been developed that are more toxic for use in chemical warfare. These nerve agents, such as soman (GD), sarin (GB), tabun (GA), cyclosarin (GF) and VX and Russian VX (VR), have already been used in the Iraq-Iran war (1983-1988) and in terrorist attacks in Matsumoto (1994) and Tokyo (1995).²

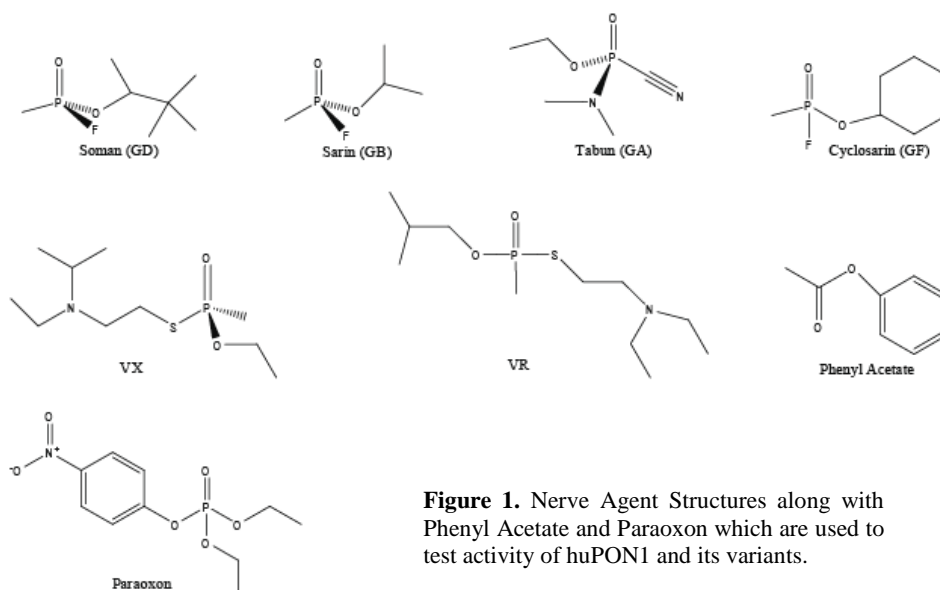


Figure 1. Nerve Agent Structures along with Phenyl Acetate and Paraoxon which are used to test activity of huPON1 and its variants.

Pharmacological treatment for these OPs has not been very effective to date, mainly because treatment is done post-exposure. Some therapies that are being utilized are anticonvulsants, acetylcholine receptor antagonists and oximes, but they are not able to directly detoxify the nerve agents *in vivo*.³ Atropine sulfate, an anticholinergic drug, has been one of the main treatments of OP poisoning,⁴ but only treats neurological and not systemic effects. To treat OP poisoning prophylactically, before the nerve agent reaches the synapse, would be more effective in blocking the antiacetylcholinesterase activity of OP agents.

A human protein that is capable of hydrolyzing organophosphates before, during, and after exposure has been found, although it is too slow in hydrolysis of OPs to be of therapeutic importance. In principle, a catalytic protein would be useful because it will take a small amount of enzyme to detoxify large amounts of nerve agent in circulation without being consumed, thus being able to protect against multiple exposures.³ However, challenges to create a useful therapeutic enzyme do arise. The enzyme must be produced in large amounts, has to be active against a large spectrum of OPs, must be able to be kept under various conditions without loss of activity, has to possess no immunological side effects, and needs to be delivered into the system through intramuscular or intravenous injection.²

HuPON1

The protein of interest in developing a therapeutic agent against organophosphate compounds comes from the human paraoxonase (PON) gene family. This family consists of PON1, PON2, and PON3, with PON1 being the most extensively studied due to its esterase/lactonase activity, as well as its anti-atherogenic activity.^{5, 6} Human Paraoxonase I (huPON1) is a glycosylated protein composed of 354 amino acids (43-47 kDa).⁵ The crystal structure of huPON1 has not yet been determined, and so the exact mechanism of the active site

is not known. A recombinant PON1, known as G2E6, has been engineered by randomly combining rabbit, mouse, rat, and human PON1 genes, and for this, a crystal structure has been obtained giving us an idea of the structure of huPON1. PON1 is described as a six-bladed β -propeller, with each blade consisting of four β -strands.² It is a calcium-dependent enzyme, with the central calcium required to maintain structural integrity while the “top” one is essential for enzymatic activities.^{2,3}

HuPON1 is synthesized in the liver and is bound to high density lipoprotein (HDL) particles in circulation, presumably via three α -helices located at the top of the propeller.² Because of this association with HDL, it is believed that huPON1 plays a role in protecting against atherosclerosis. This has been a much studied topic of late as it may prevent or limit oxidation of low-density lipoproteins (LDL).⁶ For our purpose, the HDL binding site is full of hydrophobic residues which may be a reason why huPON1 is insoluble when expressed in *E. coli*.

Using a microbial system to over-express proteins of interest, usually *E. coli*, has proven to be an effective method in expressing and characterizing recombinant human proteins for human therapeutic use. However, *E. coli* tends to express heterologous proteins in an insoluble form, causing the proteins to aggregate.⁷ Some conventional methods to obtain a soluble and correctly folded target protein include: low temperature expression, promoters with varying strengths, and solubility-enhancing fusion tags.⁸ An alternative method is site-directed rational mutations to improve the folding, stability, and solubility.⁸ We have combined the conventional strategy of using fusion tags, green fluorescent protein (GFP) and maltose binding protein (MBP), with rational engineering of huPON1 in order to obtain greater solubility.

frGFP Fusion

Green fluorescent protein (GFP) fusion can be used to report the folding of the protein of interest. The protein of interest is expressed as an N-terminal fusion with folding reporter GFP (frGFP), and if the target protein folds correctly, a chromophore will form from GFP and fluoresce in the green.⁹ If the target protein does not fold correctly, then GFP will not fold and no fluorescence will be seen (Figure 2). Waldo and co-workers expressed a test panel of 20 proteins and examined their solubilities by SDS-PAGE before measuring the whole-cell fluorescence of these test proteins as GFP fusions. The results show that the folding of GFP and formation of the chromophore is directly related to the folding of the upstream protein. The

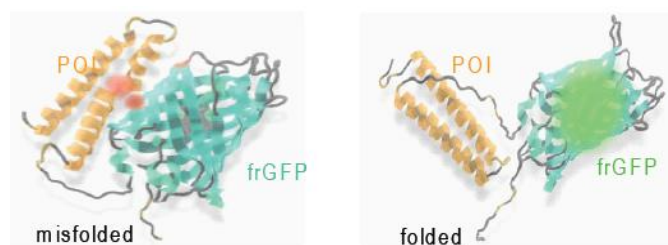


Figure 2. If the protein of interest misfolds, then frGFP will misfold and there will be no chromophore formation. If the protein of interest folds correctly, GFP will fold correctly and there will be fluorescence.¹⁰

frGFP was then used to isolate soluble variants of proteins that aggregate when expressed in *E. coli* to show a fluorescence signal that is proportional to the amount of correctly folded protein.^{9, 10} Using this reporter method, we can quickly find which rational design mutants of huPON1 are soluble when over-expressed in *E. coli*.

MBP Fusion

Soluble fusion tags have been shown to help decrease the aggregation of insoluble chimeric proteins when over-expressed in bacteria and eukaryotic systems.¹¹ When compared with two other soluble fusion partners, maltose binding protein (MBP) proved to be far more effective in solubilizing the target protein.¹² In order to see which soluble fusion protein gave the greatest overall solubility, Waugh and co-workers compared three soluble fusion tags:

thioredoxin (TRX), glutathione S-transferase (GST), and maltose-binding protein (MBP). MBP was fused to the N-terminal of six different insoluble proteins (TIMP, p16, E6, CAT Δ 9, GFP, TEV) when

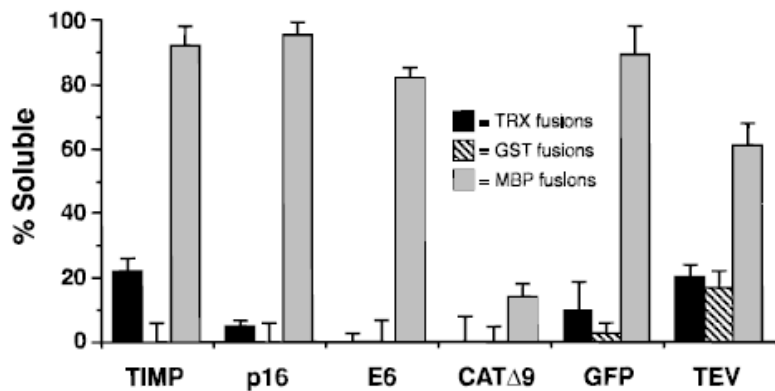


Figure 3. Comparison of solubilizing fusion tags TRX, GST, and MBP.¹²

over-expressed in *E. coli*. Each of the proteins were shown to have a greater solubility when expressed, and had a greater overall solubility than those fused with TRX and GST (Figure 3). The MBP fusion tag can be a worthwhile method in enhancing the solubility of our rational design mutants of huPON1 in *E. coli*.

Molecular Chaperones

When trying to express human proteins in *E. coli*, they often become misfolded and deposited as insoluble inclusion bodies.¹³ In order to help achieve a more stable huPON1 variant when expressed in *E. coli*, molecular chaperones can be co-expressed with our protein. R. John Ellis defines molecular chaperones as, “proteins that assist the correct non-covalent assembly of other polypeptide-containing structures *in vivo*, but are not components of these assembled structures.”¹⁴ Protein folding attempts to bury hydrophobic residues in the interior of the protein while retaining polar or charged residues on the surface. Chaperones act by helping direct the proper folding of the

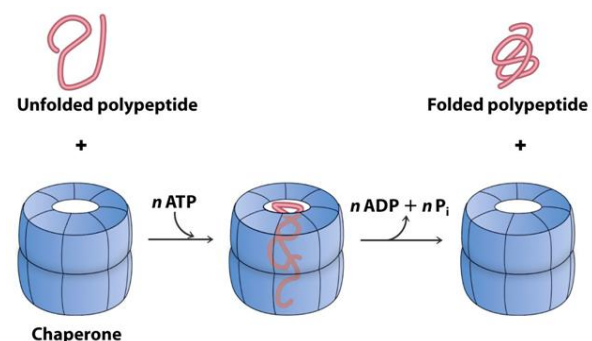


Figure 4. Chaperonin, a type of molecular chaperone that brings unfolded proteins into the cavity where the hydrophobic environment encourages proper folding.¹⁵

protein. They can reform the protein when it aggregates; allowing it to then refold properly, and by binding to hydrophobic surface residues can help prevent further misfolding.^{14, 15} These hydrophobic surfaces can interact with similar surfaces in other proteins being expressed, leading to aggregation. These molecular chaperones can be used to help refold huPON1 after expression in *E. coli*.

Objective

Increase Solubility and Stability of huPON1

Expressing recombinant proteins in *E. coli* is a very efficient and cost effective method of producing human proteins for therapeutic use. Wild-type huPON1 is able to be expressed in *E. coli* but with minimal to no protein in the soluble fraction. Because of this, only a small amount of activity was reported for huPON1 after expression and purification. The goal is to obtain a variant that closely resembles the human protein sequence in order to avoid an immune reaction when used as a therapeutic agent. The idea of rational design of protein surface amino acids will be used to change hydrophobic residues on the surface into polar ones. Two such mutants, the first mutating 12 hydrophobic residues from the HDL binding site into polar ones with equal numbers of lysine, glutamic acid, and glutamine; the second changing 16 hydrophobic to polar residues in huPON1 corresponding to the G2E6 mutations that are polar, should give more soluble variants without changing much of the activity. Additional strategies to achieve greater solubility includes fusion of huPON1 with a solubility fusion tag, Maltose Binding Protein (MBP), deletion of the unstructured N-terminal hydrophobic residues in the mutants, and use of bacterial chaperones to help with the folding and stability.

Methods

Rational mutations on HDL binding site of huPON1

Gene Construction.

Oligonucleotides were purchased (Sigma-Genosys) to synthesize a gene with mutations of Phe24Glu, Tyr185Glu, Phe186Gln, Ile187Lys, Tyr190Lys, Leu191Gln,

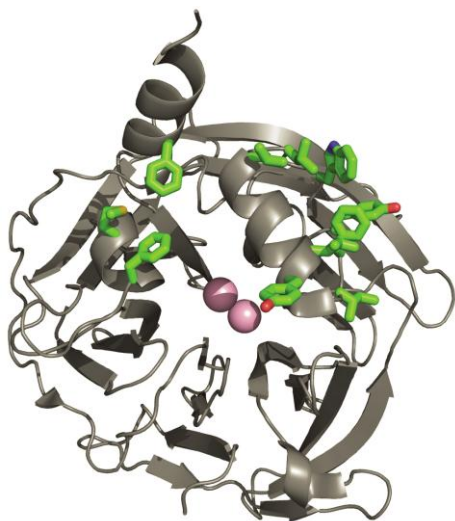


Figure 5. Mutations shown (in green) that were made on the HDL binding site

Trp194Lys, Leu198Glu, Leu200Gln, Trp202Lys, Met289Gln, Phe293Glu. The huPON1 variant with the mutations on the HDL binding site was constructed using a three fragment overlap PCR method. Each fragment was designed to complementarily overlap with each other. Once each fragment was made (Fragments 1, 2, and 3), they were mixed together and the flanking primers, 5' forward and Fragment 3 reverse, were added. Another PCR was run to extend and amplify the entire gene. The

oligonucleotides used to form these twelve mutations are shown in Table 1.

Table 1. *Primers used for huPON1 HDL mutant gene construction*

Primer	Sequence
5' forward	ATAGATATAC ATATGGCGAA GCTGATTGCA CTCACGCTCT TGGGGATGGG ACTGGCACTC TTCAGGAACC ACC
Fragment 1 forward	GGCACTC TTCAGGAACC ACC AGTCTTC AgaaCAAACA CGACTTAATG CTCTCCGAGA
Fragment 1 reverse	CATCTCC TTGGATTGCT GCT TGGGGTC TTTTGCTCG TGATCATTTG TGCCATAAAAGTG
Fragment 2 forward	agcagCAATC CaagGAGATG TATgagGGTc aaGCGaaaTC GTATGTTGTC TACTATAGTC
Fragment 2 reverse	CATACTCGAAGATTTTTTGTCCGTTAGGATGGCATCCAACCCAAAGG

Fragment 3 forward	CT AACGGACaaA AAATCTTCga gTATGACTCA GAGAATCCTCCTG
Fragment 3 reverse	GCCCTTGCTCACGAATTCGCCGCCGCTTCC

Rational G2E6 polar mutations in huPON1

Gene Construction.

The second solubilizing variant was constructed by total gene synthesis using the TBIO method.¹⁶ Hydrophobic to polar residues were mutated from G2E6 to wild-type huPON1 using 30 primers containing 16 point mutations. Fifteen of the mutations (I5T, N19R, Q21K, L31H, N78D, N80D, S81K, P82S, L96S, G101E, A137S, Q192K, Y197H, N265D, and N309D) were on the surface and an N166S compensatory mutation of Q192K were made. The full length huPON1-G2E6 polar gene was amplified using two flanking primers shown in Table 2.

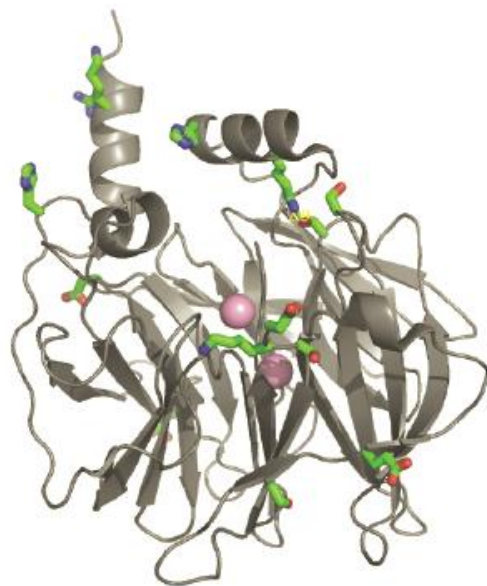


Figure 6. Polar residue mutations shown (in green) that were mutated from G2E6 to huPON1

Table 2. Primers used for huPON1 G2E6 Polar mutant gene construction

Primer	Sequence
Forward Terminal Primer	GTTTAACTTTAAGAAGGAGATATACATATGGCAAAGCTGACCGC
Reverse Terminal Primer	TGAAAATACAGATTCTCACCGCCGGTACCTAATTCACAG

Cloning mutants into pET11a-Null-frGFP vector

The huPON1 variants: huPON1 HDL and huPON1 G2E6 polar, and the wild-type huPON1 genes along with the pET11a-Null-frGFP vector with TEV- and His₆-sites were digested with NdeI and EcoRI. After overnight ligation at 16 °C, the plasmid with our gene of interest was transformed into electrocompetent DH10B cells. These cells were recovered, plated on LB

ampicillin plates and incubated overnight at 37 °C. Colonies were picked and then grown in 2YT media. The DNA was extracted (Qiagen Miniprep) and sequenced (Genewiz). Once sequencing confirmed our huPON1 mutated genes of interest, they were transformed into electrocompetent Origami B cells for protein expression.

Engineering mutants with MBP solubility fusion tag

Gene Construction and Cloning. Each mutant was amplified with primers containing restriction sites for use with pHMT-MBP vector shown in Table 3. The genes and pHMT-MBP vector were digested with NcoI and PstI. Overnight ligation at 16 °C was followed by transformation into electrocompetent DH10B cells. These were recovered and plated on LB ampicillin resistant plates and incubated overnight at 37 °C. Picked colonies were grown in 2YT media, and the extracted DNA (Qiagen Miniprep) was sequenced (Genewiz).

Table 3. Primers for MBP fusion tag

Primer	Sequence
huPON1 fw	AATAAT CCATGG GCTAA GCTGATTGCG CTCACCC
huPON1 re	AATAAT ctgcag TTA GAGTTCGCAaTAAA GAGCTTTGTGA AACACTGTGCC
huPON1 HDL fw	AATAAT gtcgac GCGAAGCTGATTGCACTCACGC
huPON1 HDL re	AATAAT ctgcag TTA GAGTTCGCAaTAAA GAGCTTTGTGA AACACTGTGCC
huPON1 G2E6p fw	AATAAT gtcgac GCAA AGCTGACCGC aCTGACTCTG TTAG GCATGG GTTTAGCAC
huPON1 G2E6p re	AATAAT ctgcag TTA TAATTCACAGTATAATGCTTTATGGAAAACCG

Re-engineering mutants with C-terminal His₆-tag

Gene Construction and Cloning. Oligonucleotides were purchased (Sigma-Genosys) containing C-terminal His₆-tag and new restriction sites NcoI and XhoI to match restriction sites on pET11a-MBP vector shown in Table 4. The genes were constructed using PCR and huPON1 HDL gene as a template.

Table 4. Primers for MBP fusion tag with C-terminal His₆-tag

Primer	Sequence
huPON1HDL fw	AATAATAAT CCATG G GCGAAGCTG ATTGCACTCA CGCTCTTGG
huPON1HDL re	AATAATAAT CTCGAG TTA ATGATGATGATGATGATG GCCGCTACTTCC GAGTTCGCAGTAAAGAGCTTTGTGAAACAC
huPON1G2E6p fw	AATAATAATCCATGG GCAAAGCTG ACCGCGCTGA CTCTG
huPON1G2E6p re	AATAATAAT CTCGAG TTA ATGATGATGATGATGATG GCCGCTACTTCC TAATTCACAGTATAATGCTTTATGGAAAACC

Expression and Purification. The expression was done with 1 L of 2YT media containing ampicillin (100 µg/mL), kanamycin (35 µg/mL), and CaCl₂ (1 mM) and grown at 37 °C. It was then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after log phase of OD₆₀₀ ≈ 0.8 was reached, and then placed at 30 °C for 6 hrs. The cells were centrifuged at 7,000 rpm for 5 min., and the harvested cell pellets were placed at -80 °C. Cell lysis was performed by resuspending the pellet using lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM imidazole, 1 mM CaCl₂, 0.1 mM MgCl₂, pH 8.0) with RNase, DNase, lysozyme, DTT (2 mM), and 10% glycerol, and then incubating on ice for 1 h. Sonication helped with the final lysis of the pellet. Tergitol (0.1%) was added and left on nutator for 2-3 hrs. The clear cell lysate was collected after centrifugation and added to 1.5 mL of Ni-NTA resin. After allowing 4 hrs, or overnight binding of the protein, the lysate was run through the column. It was then

washed with 20 mL of lysis buffer (2 mM DTT, 0.1 % Triton X-100, 10% glycerol) and then 30 mL of wash buffer (50 mM Tris-HCl, 250 mM NaCl, 40 mM imidazole, 1 mM CaCl₂, 10% Glycerol, 1 mM DTT, 0.1% Triton X-100). The protein was eluted in 10 mL of elution buffer (50 mM Tris-HCl, 50 mM NaCl, 150 mM imidazole, 1 mM CaCl₂, 10% Glycerol, 0.1% Triton X-100). The clear lysate, flow through, wash, and elution were loaded onto a 12.5% SDS-PAGE gel for analysis.

Activity Assay. A paraoxon activity assay was performed on the purified proteins by adding paraoxon (in 100% MeOH) at volumes of 0.4-4 μ L with 10 μ L of huPON1 enzyme in 50 mM Tris-HCL, 10 mM CaCl₂ buffer at pH 7.4 for a total reaction volume of 200 μ L. The hydrolysis of paraoxon was monitored at 405 nm absorbance for 30 min.

Arylesterase activity was also performed using the same procedure as above only with phenyl acetate at volumes of 0.4-5 μ L and monitored at 270 nm absorbance for 1 min.

Co-expression with Chaperones

At the same time, our lab was exploring the use of chaperones to help in protein folding. It was found that the plasmid pKJE7, containing molecular chaperones DnaK, DnaJ, and GrpE, was the chaperone system of choice for huPON1 (Magliery, Shete, Competty). The pKJE7 plasmid was co-transformed into Origami B cells with the previously mentioned huPON1 variants. The expression and purification procedure was followed as above for the re-engineered huPON1 mutants with C-terminal His₆-tag. However, chloramphenicol (30 μ g/mL) was added and L-arabinose (0.1%) was used as an inducer for the chaperones at the beginning of expression. Once achieving log phase of OD₆₀₀ \approx 8.0, IPTG (0.25 mM) was added. Activity assays were performed using procedure followed above.

N-terminal Deletion

The N-terminus of huPON1 and G2E6 contain a string of hydrophobic residues that may contribute to the insolubility of huPON1 in *E. coli*. Residues 4-17 (LIAITLL GMGLALF) in huPON1 HDL, huPON1 G2E6p, and wt-huPON, as well as in G2E6 (for comparison) were deleted in order to increase the solubility. The PCR products were cloned, expressed, and purified using the same procedures outlined above.

Results and Discussion

Rational mutations on HDL binding site of huPON1

Figure 7 shows the schematic of the three piece overlap PCR that was used to obtain the 12 mutations on the HDL binding site from hydrophobic amino acids to equal numbers of lysine, glutamic acid, and glutamine. A comparison of the HDL mutant with G2E6 to show whether the rational design achieved

greater solubility is shown in Figure 8. In terms of

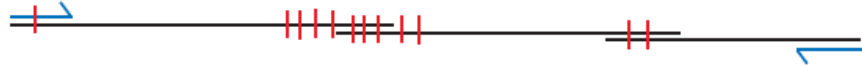


Figure 7. Three piece overlap PCR method containing all 12 hydrophobic to polar mutations.

wild-type huPON1, these 12 mutations succeeded in creating a variant with greater solubility. However, when compared with G2E6, the amount of protein expressed in the soluble fraction is somewhat less. When a whole cell fluorescence of the GFP fusions of the huPON1 variants was done (Figure 9), huPON1 HDL was the third most soluble enzyme. The deletion variant of HDL proved to be the most fluorescent, correlating into a greater solubility than the other variants, including G2E6. After testing the activity against paraoxon (Figure 10) to see if our surface mutations changed the interior chemistry, it was concluded that the HDL mutant had the greatest activity when comparing cell lysates, although down compared to G2E6. The deletion mutant,

although more soluble, was less active. This leads to the possibility that the N-terminus plays a role in determining activity.

In conclusion, rationally designing the HDL mutant led to a more soluble protein than huPON1, although less soluble than

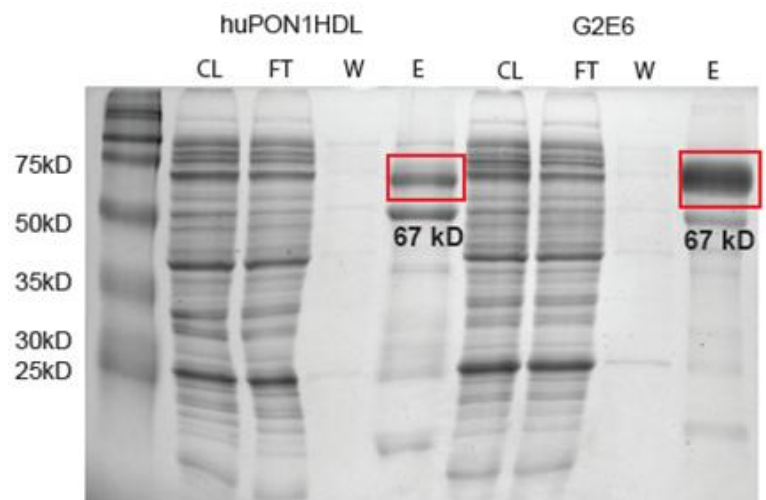


Figure 8. 12.5% SDS-PAGE gel comparing rationally designed huPON1 HDL variant with recombinant huPON1, G2E6. The clear lysate (CL), flow thru (FT), wash (W), and elution (E) of both are shown. The highlighted bands indicate huPON1 HDL and G2E6 fused with frGFP (67 kD)

G2E6. The enzyme was active against paraoxon, although less active than G2E6.

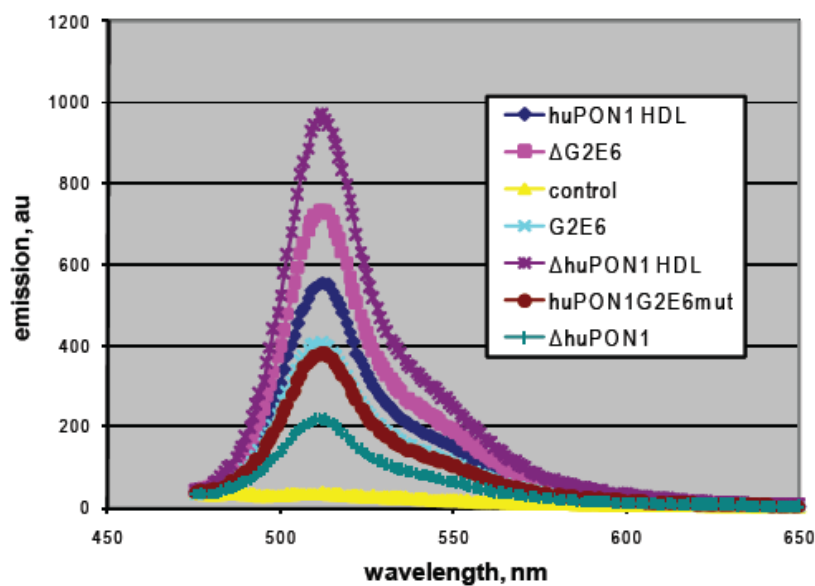


Figure 9. Emission spectra of huPON1 variants fused with frGFP expressed in *E. coli*. The solubility of the protein is highly correlated with fluorescence of *E. coli* expressing corresponding GFP fusions.

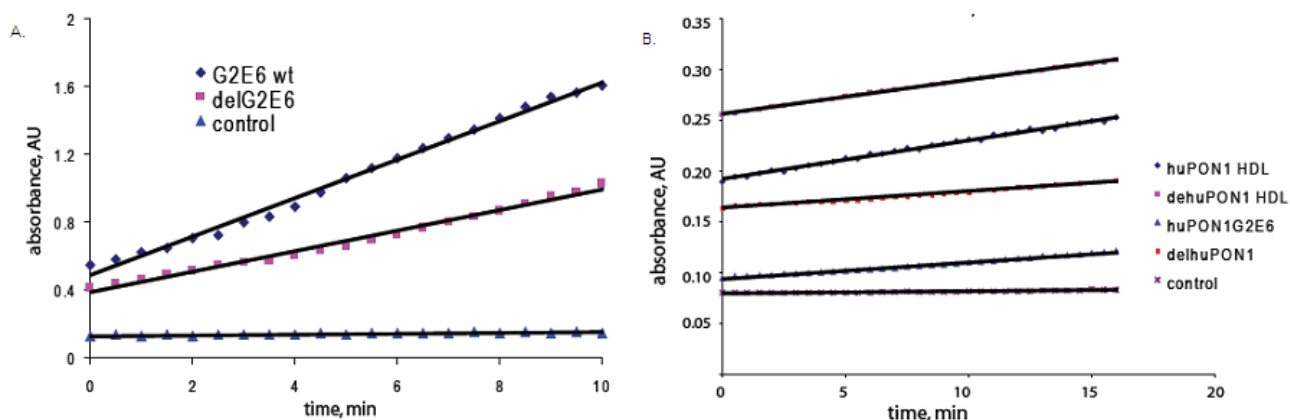


Figure 11. Cell lysate activity assay against paraoxon. These mutants were expressed in Origami B. Cells were lysed using glass beads. 20 μ L of clear lysate was used for the activity assay. A.) G2E6 and its deletion variant. B.) huPON1 variants and their deletions.

Rational G2E6 polar mutations in huPON1

The 16 mutations that are present in this variant caused the protein to become

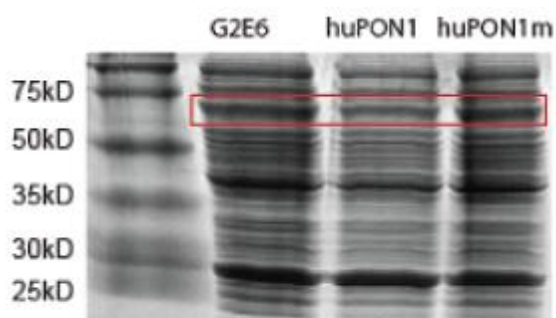


Figure 10. 12.5% SDS-PAGE gel comparing rationally designed huPON1 G2E6 polar variant with recombinant huPON1, G2E6 and wild-type huPON1. The highlighted bands indicate our proteins fused with frGFP (67 kD)

significantly more soluble when expressed in *E.*

coli as shown by Figure 11. This design, like the

HDL mutant, still produced less of a yield than

G2E6. Upon whole cell fluorescence (Figure 9),

the G2E6 polar mutant showed similar solubility

levels as G2E6. When tested against paraoxon,

we can see that the protein is still active, although

less active than G2E6 (Figure 10).

Mutants with MBP solubility fusion tag

Figure 12 shows a comparison between the HDL and G2E6 polar mutants, as well as the deletion variants of wild-type huPON1 and the HDL variant when fused with MBP. As hypothesized, the MBP fusion tag increased the solubility of all our variants a great deal. However, as can be seen by the gel, the proteins were not very pure. It appeared that other

proteins being expressed in *E. coli* were bound to the Ni-NTA resin, even after rigorous attempts at optimization. Figure 13 shows that all mutants retained their activity against paraoxon.

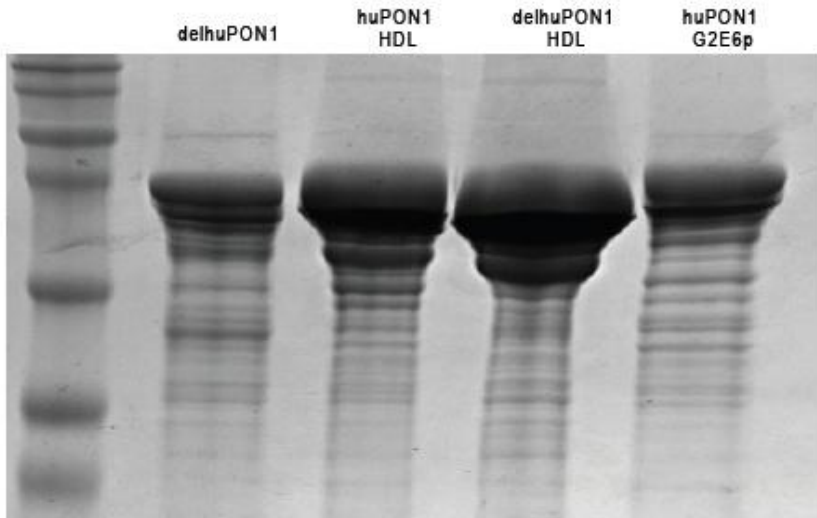


Figure 12. 12.5% SDS-PAGE gel showing the elution products of huPON1 variants fused with MBP.

The same bands were being retained on the gel consistently, even after the various purification conditions we subjected our protein to. This led to the belief that they may be truncated products, a result that can occur from over-expression of non-native proteins in *E. coli*. Because the His₆-tag was connected to

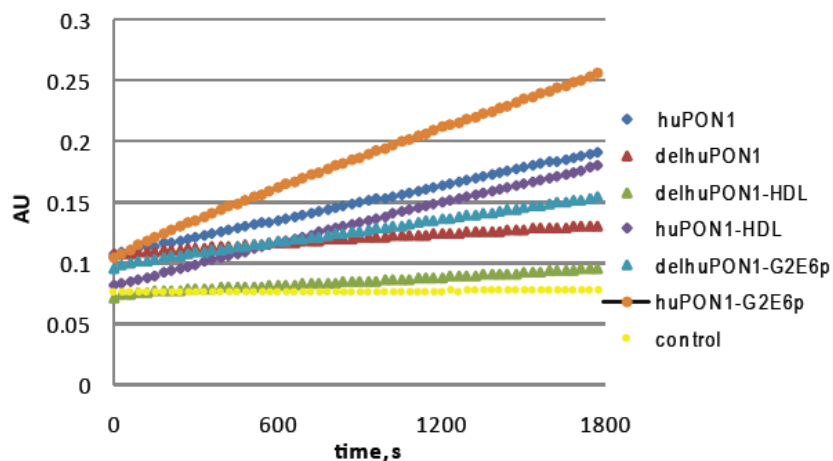


Figure 13. Paraoxon activity assay of huPON1 variants fused with MBP and expressed in Origami B. Absorbance was taken at 405 nm.

the N-terminus of the proteins, the truncated products can adhere to the Ni-NTA column. In order to definitively determine that these were in fact His₆-tagged as well, a HisProbe Western blot was run

(Figure 14), proving that the extra bands were from truncated product of our huPON1 genes. To get rid of these extra products, we reengineered the vectors to have a C-terminal His₆-tag fused to our huPON1 variants (Figure 15).

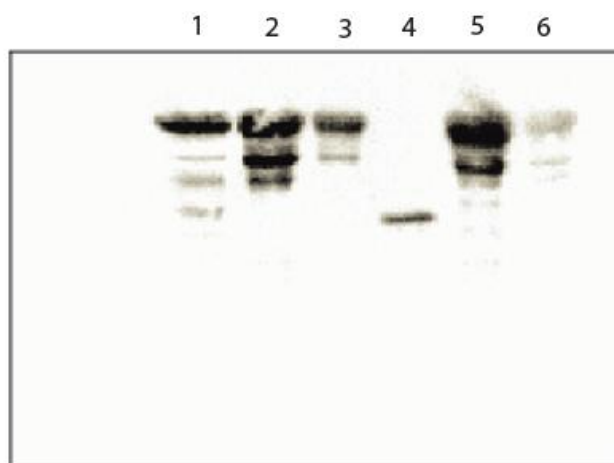


Figure 14. Western blot of HisProbe experiment of huPON1 variants fused with MBP. 1-huPON1 HDL cell lysate; 2-3 huPON1 HDL; 4-MBP; 5-huPON1 G2E6p; 6-ΔhuPON1

Reengineered C-terminal His₆-tag co-expressed with chaperones

At the same time as the reengineering of our vectors, our lab was exploring the use of molecular chaperones to help in the folding of huPON1 when expressed in *E. coli*. Out of

several different chaperone sets tested, DNA KJE7 (DnaK, DnaJ, and GrpE),¹⁷ was able to

express some amount of active wild-type huPON1. We speculated this might also be useful for our variants. The DNA KJE7 vector and the reengineered C-terminal His₆, MBP fusion huPON1 and its variants were co-transformed and expressed with cleaner bands (Figure 16) and better yields (Table 5.). All solubilizing variants were expressed in higher yields than wild-type huPON1, with the deletion variants producing more protein than their full length counterparts. All variants proved to be active against both phenyl acetate and paraoxon.

Kinetics of huPON1 and its variants

The kinetics, shown in Tables 6 and 7, of phenyl acetate and paraoxon hydrolysis of huPON1 and its variants show wild-type huPON1 to be more active compared to all of the other

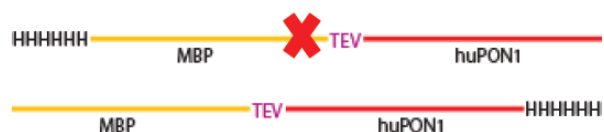


Figure 15. Scheme showing how a reengineered C-terminal His₆-tag was able to reduce the truncated products after protein purification with an N-terminal tag produced multiple eluted products

variants. The N-terminal deletion mutants were less active than any of the full length mutants, indicating that the unstructured portion of the N-terminus may play a role in either activity or stability of the protein.

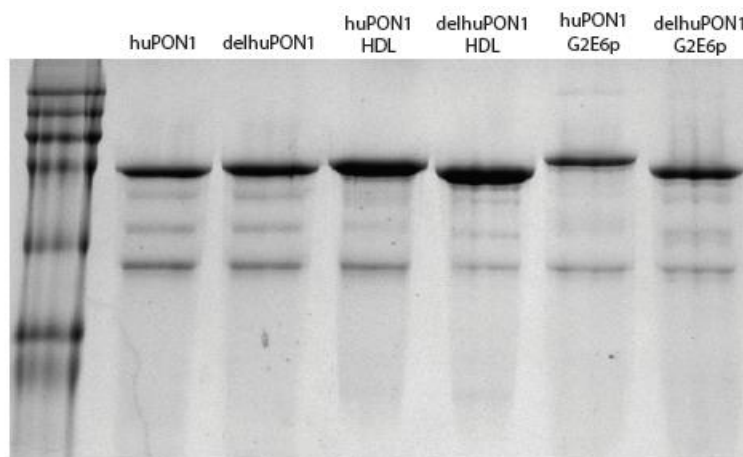


Figure 16. 12.5% SDS Page Gel showing the elution products of C-terminal His₆-tag, MBP fusion, huPON1 and its variants after being co-expressed with chaperone set DNA KJE7.

HuPON1	delhuPON1	huPON1-HDL	delhuPON1-HDL	huPON1-G2E6p	delhuPON1-G2E6P
2.00	2.40	3.70	3.90	2.70	3.20

Table 5. Concentrations (mg/L) of MBP fused huPON1 and its variants after reengineering the vector with a C-terminal His₆-tag and co-expression with chaperone set DNA KJE7.

variants	V _{max}	K _M	k _{cat}	k _{cat} /K _M
huPON1	2.33E-01	1.6045	2.87E+03	1.79E+03
delhuPON1	6.51E-02	1.9516	8.02E+02	4.11E+02
HDL	1.38E-02	0.67191	1.23E+02	1.83E+02
delHDL	2.59E-02	1.4223	1.15E+02	8.07E+01
G2E6p	2.59E-01	2.0598	2.62E+03	1.27E+03
delG2E6p	5.03E-02	1.8839	5.09E+02	2.70E+02

Table 1. Kinetics data of huPON1 and its variants on the hydrolysis of phenyl acetate in mM min⁻¹.

Conclusion

The study of huPON1 both as a catalytic bioscavenger and as an insight into how proteins function has proven to be challenging yet very rewarding. We can see by our two mutants, the HDL mutant and the G2E6 polar mutant that it only takes a small number of surface residues to largely determine the solubility of the protein. We can also see that making a small number of

surface mutations that are far from the active site still can affect the activity. Our mutants, though very soluble, had decreased activity by an unknown mechanism. This problem of expressing human proteins in *E. coli* continues to be a great challenge in biochemistry and gene engineering.

There is more than one way to achieve solubility as we have shown with our variants. The rational design by changing hydrophobic residues to polar or charged residues was a huge help in making huPON1 more soluble. However, when fused with MBP, even wild-type huPON1 was soluble. This is a large feat considering previous literature put the overall yield after expression and purification to be 5 mg from 12 L of fermentor prep, or ~450 µg per L of culture.¹⁸ Our yield of 2 mg/L from shake flask expression of huPON1 fused with MBP when co-expressed with molecular chaperones, DNA KJE7, far exceeds the previous amount. The molecular chaperones helped increase the expression yield of huPON1 and its variants by helping fold the protein correctly *in vivo*. This method also helped in the problem of truncation products. By correctly folding, the protein will less likely aggregate and form inclusion bodies. Moving the His₆-tag to the C-terminus of our protein also decreased the truncation products. If for some reason the translation of our gene terminates early, then the translation of the His₆-tag also will also be terminated. The only problem with the C-terminal tag is cleavage of it through a TEV protease site. This can, however, be changed through re-engineering of our pET11 vector.

Since we have combined multiple solubilizing techniques to express large amounts of huPON1 in *E. coli*, we now have to continue to try and improve the stability of this protein. If we can screen for stability and activity, perhaps a fully functional, highly active huPON1 can be optimized with very limited immunological effects. We can also continue optimizing the

purification conditions to obtain a more stable huPON1. In any case, working with G2E6 and our soluble variants as models, we can try and pinpoint the mechanism of hydrolysis of organophosphates so huPON1 can work as an efficient catalytic bioscavenger.

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